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Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells

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In this pilot study, we investigated the ability of autologous dendritic cells pulsed *ex vivo* with tumor-specific idiotype protein to stimulate host antitumor immunity when infused as a vaccine. Four patients with follicular B-cell lymphoma received a series of three or four infusions of antigen-pulsed dendritic cells followed, in each instance, by subcutaneous injections of soluble antigen two weeks later. All patients developed measurable antitumor cellular immune responses. In addition, clinical responses have been measured with one patient experiencing complete tumor regression, a second patient having partial tumor regression, and a third patient resolving all evidence of disease as detected by a sensitive tumor-specific molecular analysis.

Although conventional chemotherapy and radiation treatments for low-grade non-Hodgkins lymphoma often induce tumor regression, these malignancies remain essentially incurable. The ineffectiveness of these modalities, coupled with the potentially harmful effects of such treatment, has motivated the search for alternative tumor therapies. One such alternative is to utilize the immune system specifically to target and to eliminate neoplastic cells on the basis of their expression of immunogenic surface markers. B-cell malignancies are unique in that a tumor-specific cell surface antigen has been identified. These malignancies express immunoglobulin receptors, which can distinguish these neoplastic cells from normal B cells by virtue of specific idiotypic determinants[1-3]. The term idiotype refers to antigenic motifs formed by the combination of the variable regions of immunoglobulin heavy and light chains. Because these malignancies are monoclonal, all the cells of a given tumor express identical immunoglobulin receptors.

Idiotype determinants of B-cell malignancies are antigens that can be recognized by the immune system. Animal and human studies have demonstrated the effectiveness of anti-idiotype antibodies to target and kill lymphoma cells[4,5]. In some cases, such treatments have induced long-term clinical remissions. Animal studies have also demonstrated the ability of an idiotype vaccine approach to induce host immunity against tumor challenge[1,6-9] and to cure animals with established disease[10]. An ongoing clinical trial evaluating the use of idiotype vaccination in B-cell lymphoma has been successful in inducing anti-idiotype immune responses and, in some cases, tumor regression in humans[11,12]. Ultimately, the success of this treatment depends on the ability of the vaccine to stimulate a strong and uniform antitumor immune response in all patients.

Typically, vaccine approaches have incorporated foreign carrier proteins, adjuvants, cytokines[7,8], and genetically engineered viruses[13,14] in attempts to increase immunogenicity. The net effect of all these approaches is to stimulate the effective processing and presentation of antigens to the immune system. Thus, a critical target of vaccines is the specialized antigen-presenting cell, the most immunologically powerful of which is the bone marrow-derived dendritic cell (DC). DCs are present in small numbers in most tissues including skin, liver, lung, spleen, blood, lymphoid organs, and bone marrow[15]. Morphologically, they are large cells with elongated, stellate processes called dendrites. They lack cell surface markers typical for B, T, NK, or monocyte-macrophage cell lineages[15], but express high levels of MHC class I and II as well as the immunomodulatory proteins B7-1 (CD80), B7-2 (CD86) (refs 16, 17), CD40 (refs 18, 19), and two adhesins, the intercellular adhesion molecule ICAM-1 (CD54), and the lymphocyte function-associated protein LFA-3 (CD58) (ref. 19). They are also capable of producing the interleukin IL-12, a potent cytokine that induces the development of interferon-γ-producing cells[20]. DCs pulsed (cocultured) with proteins are capable of presenting processed antigen for days[21,22]. *In vitro*, these cells are capable of stimulating naive T cells to recognize and to respond to a variety of antigens[23-25]. They are powerful stimulators of immunity when administered as a vaccine to animals[22,26], even for very weak antigens[27]. In animal models of lymphoma, a single immunization with idiotype protein-pulsed DCs is capable of inducing protective immunity against tumor challenge[26].

Recently, methods have been developed for the isolation of human dendritic cells from peripheral blood[23,28]. A clinical trial was initiated to evaluate the efficacy of tumor-specific idiotype protein-pulsed autologous DCs in the treatment of B-cell lymphoma by using this new technology. We report here on the results in four patients treated with this form of active immunotherapy.

Vaccinations

Tumor biopsies were obtained from patients with B-cell lymphoma, and the immunoglobulin (idiotype protein) produced by each tumor was obtained by cell fusion techniques[29]. DCs were isolated from the peripheral blood of patients with lymphoma by

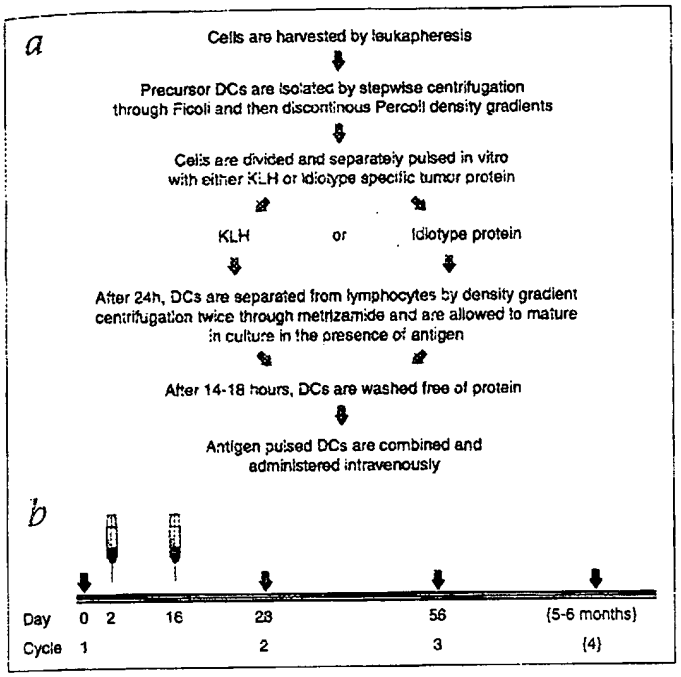

Fig. 1 Preparation and administration of DC vaccine. *a*, Preparation of antigen-pulsed dendritic cell vaccine. *b*, Immunization schedule. Each vaccine cycle (bold arrow) consisted of the collection, antigen-pulsing, infusion of DCs, and the subsequent protein injections. Patients underwent leukapheresis on day 0 to harvest mononuclear cells. Dendritic cells were purified and pulsed *in vitro* with antigen and then reinfused on day 2. On day 16, patients received subcutaneous injections of either KLH or idiotype protein in saline in separate sites. Three primary vaccinations were given every 28 days, and a fourth treatment was given 5–6 months later.

leukapheresis and density gradient centrifugation (Fig. 1*a*). The cells were divided and incubated separately with either idiotype protein or KLH (keyhole limpet hemocyanin), an immunogenic protein that served as a control for the immunocompetence of each patient. During the isolation procedure, DCs were not exposed to foreign proteins. As outlined in Fig. 1*b*, each patient received three immunizations consisting of the infusion of autologous antigen-pulsed DCs followed two weeks later, in each instance, by injections of idiotype protein or KLH in saline in separate subcutaneous sites in accordance to a schedule established in an animal model[26]. The soluble antigens were given in order to boost the primary response induced by the DC infusion. A fourth DC infusion and protein immunization was given five to six months later.

Three patients with follicular low-grade lymphoma have completed the planned four vaccinations; one patient has completed three treatments. Prevaccination clinical characteristics of each are shown in Table 1. Despite extensive chemotherapy, patients D1, D2, and D4 had measurable tumor still present before beginning immunization. Patient D3 had equivocal radiographic findings but was found to have evidence of disease in bone marrow (BM) and blood by a tumor-specific molecular analysis.

The number of DCs infused with each treatment ranged from 2 to 32 million cells (median 5 million). The total yield of DCs was four times as high in patient D1 as in the other patients. Patients D2 and D4 were leukopenic (white blood cells (WBCs) 2,600–3,000/μl, normal range: 4,000–11,000/μl) after completion of chemotherapy, and this persisted into their DC treatments. The average dose given to patients D2, D3, and D4 was only 4.3 million cells and appeared to reflect the lower WBCs in these patients. However, despite the relatively low doses of DCs given and the leukopenia, antitumor immune responses were induced in all patients.

All treatments were well tolerated, and no significant side effects were associated with the infusion of antigen-pulsed DCs. By the second vaccination, all patients developed mild, transient erythema at the site of their subcutaneous KLH injection, which resolved after 48–72 hours.

Immunologic responses

Blood samples were collected for immunologic testing before each vaccination and during each follow-up visit. All four patients developed strong humoral and peripheral blood mononuclear cells (PBMCs) proliferative responses against the control KLH protein (Table 2). In patients D2, D3 and D4, these responses were measured within one or two weeks following the first infusion of antigen-pulsed DCs and before receiving any soluble protein injections (Fig. 2). Cellular proliferative responses against tumor idiotype protein developed in all patients. These responses were specific for the autologous tumor idiotype when compared to irrelevant, isotype-matched immunoglobulins. An anti-idiotype response was measured after the first vaccination in patient D4 and after two or three vaccinations in the other patients. The level of intensity of anti-idiotype and anti-KLH cellular re-

Table 1 Prevaccine patient characteristics

Patient characteristics	D1	D2	D3	D4
Age	59	44	34	45
Gender	F	F	F	M
Histology	FM	FM	F/DSC	FSC
Tumor isotype	IgG λ	IgM κ	IgG κ	IgG λ
Disease sites	Paracardiac, mediastinal	Periaortic, parailiac	Equivocal findings: BM, periaortic, mesenteric	Periaortic, parailiac axiliary
Prior treatment	Daily Chl. × 18 months, pulse Chl. × 2, Chl/VCR/Bleo × 4	Daily, pulse Chl. × 4 months CVP × 10	CVP × 7 Fludarabine × 3	CVP × 9 Fludarabine × 5
Interval from chemotherapy to vaccine (months)	24	2	2	3

Histology: FM, follicular mixed small cleaved and large cell lymphoma; FSC, follicular small cleaved cell lymphoma; F/DSC, follicular and diffuse small cleaved cell lymphoma.
Treatment: Chl., Chlorambucil; VCR, vincristine; Bleo, bleomycin; CVP, cylophosphamide, vincristine, prednisone.

Table 2 Immune response

Patient	Anti-KLH		Anti-idiotype	
	Ab	Cell	Ab	Cell
D1	+	+	–	+
D2	+	+	–	+
D3	+	+	–	+
D4	+	+	–	+

Ab, humoral response; cell, PBMC proliferative response.

sponses has been similar in all of our patients. A plateau was usually seen after the second or third immunization. Sustained responses have been measured for several months after completion of therapy in our patients, though the intensity declines with time. In patients who have received a fourth vaccine, the immune response is boosted. No specific anti-idiotype antibody responses were detected.

Peripheral blood mononuclear cells from patient D1 were expanded in culture in the presence of idiotype protein and tested for their ability to lyse autologous tumor hybridomas in a ^{51}Cr release assay (Fig. 3). At the highest effector-to-target ratio, 33% tumor lysis was observed against the autologous tumor hybridoma target and 9% against an isotype-matched, unrelated hybridoma. No cytotoxicity was noted against the hybridoma partner or, in a separate experiment, against autologous Epstein–Barr virus-transformed B cells.

Clinical responses

Case 1. Patient D1 was initially diagnosed with stage IIIA follicular lymphoma. She was treated with several courses of chemotherapy with the last dose given in February 1991. She later recurred and had slowly progressive paracardiac and periaortic disease documented by serial radiographic studies. Vaccine treatments with autologous, idiotype-pulsed DCs were initiated in May 1993. Three months after completing the three primary vaccinations, restaging CT scans demonstrated significant regression of her paracardiac and periaortic disease. The patient later received a fourth treatment. Restaging CT studies 10 and 21 months after the completion of the three primary immunizations demonstrated the patient to be in a clinical complete remission (Fig. 4).

Case 2. Patient D2 had residual lymphoma in periaortic and parailiac regions. Despite having made a measurable anti-idiotype proliferative response, she subsequently developed progressive disease four months after the primary series of vaccinations. A fourth immunization was given. The patient has remained stable and has not required further therapy.

Case 3. Patient D3 had equivocal prevaccine radiographic findings of mesenteric and periaortic adenopathy measuring up to 1.5 cm at sites of previously bulky disease. BM biopsies revealed atypical lymphoid aggregates which were not diagnostic of lymphoma; however, a sensitive molecular analysis demonstrated evidence of disease in marrow and blood. Restaging CT studies seven months after completion of the primary three vaccinations demonstrated no significant change. Repeat BM biopsies were negative histologically and by molecular analysis (Fig. 5).

Case 4. Patient D4 had stage IVA follicular lymphoma with growing disease noted before initiation of vaccine therapy. The patient recently completed three of the planned four treatments and already has had a minor response with regression of some peripheral lymph nodes.

Patients D2 and D4 were leukopenic during treatment. At this time, the role that leukopenia or DC dose might have in the overall clinical response remains unclear.

Detection of minimal disease

A semi-nested PCR technique capable of detecting the tumor-specific immunoglobulin heavy chain gene rearrangement was applied to patient D3. The sensitivity of this cDNA based assay to detect tumor cells was repetitively determined by dilutional analysis to be 1 tumor cell in the presence of 10^7 normal PBMCs. Samples of BM and PBMC pre- and postvaccine treatments were examined for the presence of tumor (Fig. 5*b*.). Tumor-specific DNA was found in prevaccine samples of BM and PBMCs. At the level of detection of this assay, no tumor was found in the samples collected after completion of vaccine treatments. The PCR products were confirmed to be specific for tumor by directly sequencing the bands seen in Fig. 5*b*. A similar analysis was performed on PBMCs collected on patient D1 and no tumor was found. However, in this case, prevaccine samples were not available for comparison (data not shown).

Discussion

In this study, we have examined the ability of autologous DCs pulsed with idiotype protein to function as a vaccine when infused intravenously. In all four patients, humoral and cellular responses developed against the control immunogen KLH, and these responses occurred as early as one to two weeks after a single infusion of DCs. This contrasts with our experience using an idiotype-KLH-adjuvant vaccine in which anti-KLH cellular re-

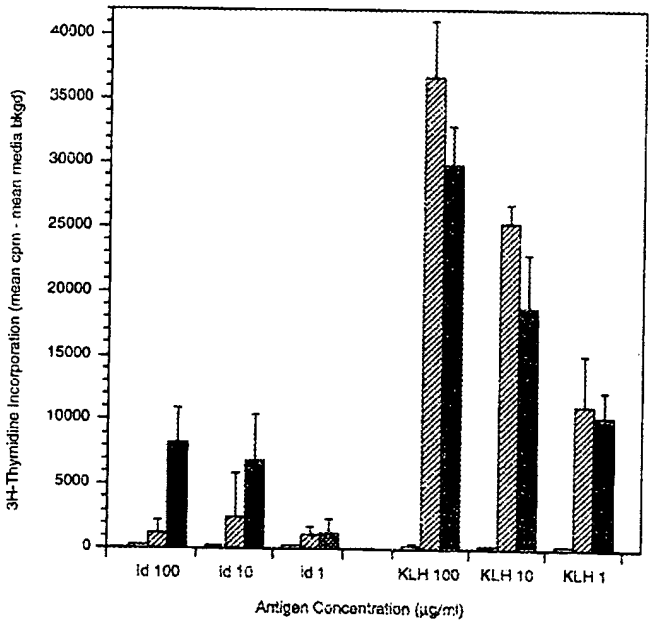

Fig. 2 Peripheral blood mononuclear cell proliferative response. PBMCs of patient D4 were cocultured *in vitro* with concentrations of KLH or Id protein varying from 0 g/ml to 100 µg/ml. Incorporation of [^{3}H]thymidine is reported as the mean c.p.m. of quadruplicate samples above the media background with error bars representing standard deviations. PBMCs were obtained pretreatment (□), two weeks after the first infusion of DCs before the soluble protein boost (▨), and two weeks following the second infusion of DCs (■). No response was seen pretreatment. Incorporation of [^{3}H]thymidine in response to an irrelevant, isotype matched control protein was ~2,000 c.p.m. two weeks after the second infusion of DCs.

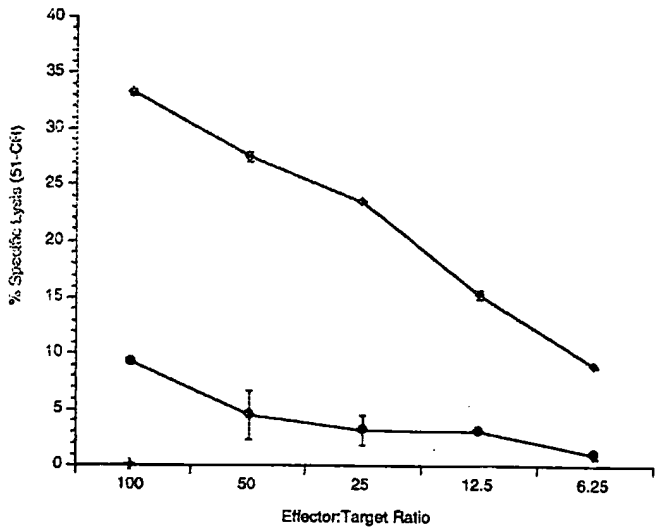

Fig. 3 Antitumor cytotoxic effector cells from an immunized patient. PBMCs were expanded *in vitro* in the presence of soluble idiotype protein and IL-2 over five weeks. Effector cells were then tested for their ability to lyse 1×10^4 ^{51}Cr-labeled targets at various effector:target ratios. The targets included the autologous tumor hybridoma of patient D1 (◆), an isotype-matched, unrelated hybridoma (●), and the hybridoma partner K6H6B5 (+). T cells could not be expanded against an irrelevant, isotype-matched protein. Data are shown as the means of duplicate samples with error bars representing the actual data points.

sponses usually require two or three immunizations to occur (unpublished observations). Significant antitumor idiotype PBMC proliferative responses developed in all patients. Though an anti-idiotype response was seen after the first vaccination in one patient, these responses generally occurred after two or three DC infusions and may reflect the relatively weak immunogenicity of these tumor proteins. These immune responses were induced with as few as two to three million DCs, attesting to the potency of this form of treatment. No anti-idiotype antibody responses were detected in this study. All of our patients had residual disease, and one possibility is that anti-idiotype antibody is bound to residual tumor or to circulating tumor idiotype protein. In this scenario, anti-idiotype antibodies would not be measurable by our assays. A second possibility is that the dendritic cells are stimulating primarily a T_H1 cell response, and cellular immune responses are primarily being induced.

The predominance of anti-idiotype cellular immune responses differs from the findings of our idiotype-KLH-adjuvant vaccine trial where anti-idiotype antibodies are more often induced (unpublished observations) and appears to distinguish the effects of these two methods of immunization. Combining these two forms of vaccination, idiotype protein-pulsed DCs and idiotype-KLH conjugated protein in adjuvant, may ultimately result in improved antitumor T-cell and antibody responses and, potentially, even greater clinical effects. By themselves, the subcutaneous protein immunizations used in this trial would not be expected to induce immune responses. As demonstrated in a separate study, the treatment of patients with three vaccinations of idiotype-KLH in the absence of adjuvant failed to induce immunity to the tumor protein[12].

Clinical responses were observed in this study. Patient D1 underwent a complete tumor regression in association with the development of measurable immunity and remains in clinical remission at this time. Patient D3 had equivocal radiographic findings of disease which did not change following vaccine treatments; however, tumor cells found in BM and blood by a sensitive molecular test before vaccine therapy resolved with the development of immunity. Patient D4 recently completed three vaccinations and has already demonstrated a minor response with regression of some nodal sites.

This study has demonstrated the ability of antigen-pulsed DCs to stimulate clinically relevant immune responses in humans. DC precursors used in our study are exposed immediately after isolation to tumor protein. Previous studies have demonstrated that immature DCs are more efficient in their ability to process whole protein antigens than are cultured mature DCs (refs 22, 30, 31). These antigen-pulsed cells then mature in tissue culture and develop high levels of cell surface MHC class I and II and the costimulatory molecules B7-1 and B7-2 characteristic of mature DCs (ref. 23 and unpublished observations). The exposure of DCs to high concentrations of purified tumor antigen, in combination with the maturation process, may explain why the *ex vivo* loading of these cells

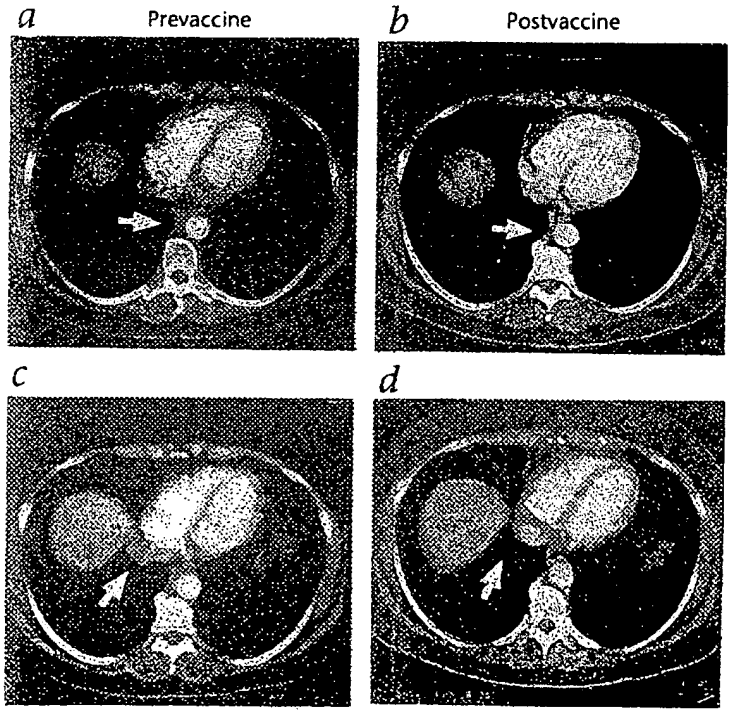

Fig. 4 Clinical response to vaccine therapy. *a* and *c*, Prevaccine CT scans through the chest of patient D1. The arrows point to periaortic lymph nodes (*a*) and a paracardiac mass (*c*). *b* and *d*, represent equivalent CT scans 10 months after completion of the initial three vaccine treatments. All sites of disease have resolved, and the patient has remained in remission at the time of restaging studies 24 months after beginning treatment.

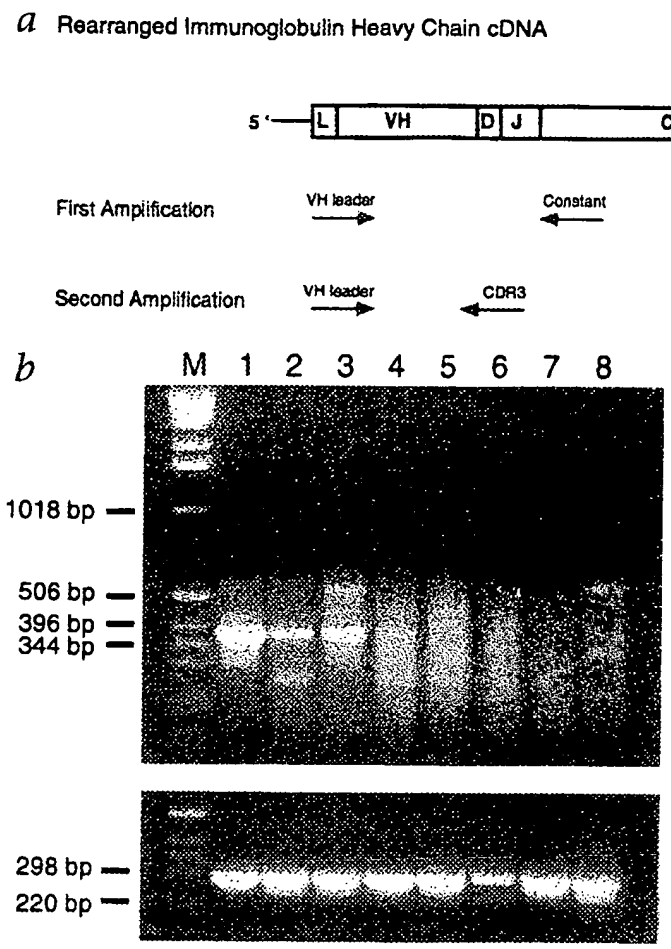

Fig. 5 Detection of residual disease using a tumor-specific PCR analysis. *a*, First-round amplification of immunoglobulin heavy chain cDNA of this semi-nested PCR analysis utilized a sense primer to the leader segment of the variable region gene and an antisense primer to the constant region corresponding to the tumor. In the second amplification, the products of the first PCR were reamplified using the same leader primer and a unique tumor-specific antisense primer corresponding to the CDR3 region of the heavy chain gene. *b*, The upper gel shows the semi-nested PCR analysis of clinical samples from patient D3. Lane M is a DNA size ladder. Lane 1 is the tumor-specific second amplification of cDNA from tumor of patient D3; lane 2, from the dilution of 1 tumor cell into 10^7 normal PBMCs; lane 3, from prevaccine BM cells; lane 4, from prevaccine PBMCs; lane 5, from postvaccine BM cells; lane 6, from postvaccine PBMCs; lane 7, from normal spleen cells; and lane 8, from an isotype and V_H gene family matched, unrelated tumor. The tumor-specific band at ~370 bp is seen in prevaccine samples of BM and PBMCs but not in postvaccine samples. The faint band at ~500 bp represents carryover from the first PCR reaction. As shown in lane 2, the level of detection was 1 tumor cell in the presence of 10^7 normal PBMCs. Bands in the lower gel represent PCR amplification of β_2-microglobulin and demonstrate the integrity of each cDNA sample.

with antigen is effective in stimulating an immune response when the presence of *in vivo* tumor is not.

The use of DCs as a vaccine is an area of intense investigation. The *in vitro* priming of DCs allows a unique opportunity to modulate the resulting immune response, and in the future, it may be possible to enhance antitumor immunity through the direct application of cytokines and other factors. The vaccine approach described here could be applied to other diseases. The MAGE, BAGE, Mart-1 antigens associated with melanoma[32–34], the MUC-1 antigen associated with various carcinomas[35], and the various glycoproteins of HIV are all potential targets. It may be possible to eliminate the need for leukapheresis. The combination of granulocyte-macrophage–colony-stimulating factor (GM-CSF) with tumor necrosis factor-α (TNF-α) or IL-4 has been reported to expand DCs in culture from small volumes of peripheral blood[36–38]; however, it is not clear at this time whether such expanded cells will function equivalentiy in their ability to process and present antigen or to stimulate cellular immune responses as the freshly isolated cells used here.

Our results indicate that DCs can be safely harvested, pulsed with antigen and infused repeatedly into humans with no significant toxicity. Small numbers of antigen-pulsed DCs are effective in stimulating immune responses after a single treatment. In addition, the antitumor immunity induced by these vaccine treatments have been associated with significant tumor regression in two cases and the resolution of molecular evidence of disease in another. Therefore, antigen-pulsed DCs represent a powerful new vaccine capable of inducing cellular immune responses against weak antigens such as tumor proteins. This approach may be generally applicable in the treatment of B-cell malignancies and is limited only by the effort required to make idiotype proteins for each patient.

Methods

Patients. Patient characteristics are shown in Table 1. All four patients had follicular low-grade lymphoma and had previously been treated with a variety of chemotherapy regimens. Patients D1, D2 and D4 had measurable residual disease before beginning vaccine therapy. Patient D3 had equivocal radiographic evidence of mesenteric disease and atypical histologic findings in BM.

Antigens. Lymph node biopsies were obtained before chemotherapy, and tumor cells were fused to the cell line K6H6B5 as previously described[29]. The resulting hybridomas were initially screened by an enzyme-linked immunosorbent assay (ELISA) for the production of immunoglobulin matching the isotype of the tumor. Then, the immunoglobulin heavy chain variable region (V_H) gene of each hybridoma was amplified and sequenced[39]. Hybridomas were confirmed to be derived from the tumor when the sequence corresponding to the third complementarity-determining region (CDR3) of the heavy chain gene matched that of the original tumor. The idiotype protein was purified from hybridoma culture supernatants by affinity chromatography (Protein A for IgG and anti-IgM antibody columns for IgM).

Keyhole limpet hemocyanin (KLH, Calbiochem, La Jolla, California) was depleted of endotoxin as previously described[12] and used as a nonspecific control vaccine.

Dendritic cell preparation. Patients underwent leukapheresis using a COBE cell separator. Peripheral blood mononuclear cells (PBMCs) were first collected by separation through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and then monocytes were removed by centrifugation through discontinuous Percoll (Pharmacia) gradients as previously described[25,28] (Fig. 1*a*). Monocyte-depleted PBMCs were divided into two samples and placed separately into media (RPMI 1640, 10% autologous serum) containing either idiotype protein or KLH at a concentration of 2 μg/ml. After 24 h of culture in a humidified incubator at 37 °C supplemented with 10% CO_2, DCs were separated from high-density lymphocytes by sequential centrifugation through 15% and then 14% (wt/vol) metrizamide gradients (Sigma); after which, the enriched, low-density DCs were cultured again for 14–18 h in media supplemented with 50 μg/ml idiotype protein or KLH. The purity of DCs was confirmed by morphology, and when adequate numbers of cells were available, by flow cytometry staining using anti-CD14, CD19, CD3, and HLA-DR. Preparations contained between 50 and 90% dendritic cells. Contaminating cells consisted of T cells and monocytes. After incubation, the cells were washed free of antigen, and the two populations of DCs were combined into 100 ml of saline containing 5% autologous serum. Between 2 and 32 million DCs were collected on each occasion, representing approximately 0.08 to 0.4% of the starting mononuclear cell population. Patients were premedicated with 650 mg acetoaminophen and 25 mg diphenhydramine, and infusions of DCs were administered intravenously over an hour.

ELISA measurement of humoral responses. Idiotype protein or isotype-matched immunoglobulins were captured onto microtiter plates coated with goat antihuman heavy chain antibodies (BioSource International, Camarillo, California). When the tumor idiotype was an IgG, F(ab')$_2$ fragments were produced by digestion with immobilized pepsin (Pierce, Rockford, Illinois) and used to coat microtiter plates directly. Pre- and post-immunization patient serum samples were serially diluted and allowed to bind to the target proteins. The binding of anti-idiotype antibodies was detected by polyclonal goat anti-human IgG antibodies labeled with horseradish peroxidase (HRP). A response was interpreted as positive when a fourfold rise in anti-idiotype antibody titer was found when compared with prevaccine serum and hyperimmune serum binding of irrelevant isotype-matched proteins used as specificity targets. Antibody responses to KLH were measured by directly coating microtiter plates with KLH and allowing patient serum to bind. Anti-KLH antibodies were detected with KLH labeled with HRP.

Cellular proliferation assay. PBMCs were cultured in media containing KLH, idiotype protein or isotype-matched, irrelevant protein at concentrations of 0 to 100 μg/ml as previously described[12]. Determination of [^{3}H]thymidine incorporation was performed in quadruplicate on days 5 to 6. A response was interpreted as positive when incorporation of more than two times background was found on two or more occasions.

Cellular cytotoxicity assay. PBMCs obtained after completion of all vaccine treatments were stimulated with autologous tumor idiotype protein at a concentration of 50–100 μg/ml and expanded in the presence of IL-2. Cells were restimulated every 10–14 days with antigen and autologous PBMCs as feeders. After ~5 weeks of *in vitro* expansion, the cells were assayed for their ability to kill various tumor targets in a standard 4-h ^{51}Cr release assay. Targets consisted of a hybridoma made from patient D1 tumor cells, a hybridoma from an irrelevant, isotype-matched tumor, the K6H6B5 hybridoma partner, or autologous Epstein–Barr virus-transformed PBMCs from patient D1. Targets were labeled with ^{51}Cr as previously described[40], and 10^4 labeled cells were plated with various concentrations of effector cells. Percent cytotoxicity is reported as the [(c.p.m. – spontaneous release) ÷ (total c.p.m. – spontaneous release)] × 100%.

Measurement of residual disease using a tumor-specific CDR3 analysis. The sequence of the unique immunoglobulin V_H gene CDR3 segment expressed by each tumor was determined using methods previously described[39]. Antisense CDR3 specific primers were used in a semi-nested PCR to evaluate patients for residual disease (Fig. 5*a*). PBMC or BM aspirate samples were collected, and 10^7 cells were used to generate cDNA[39]. First-round amplification of 1/20th of patients cDNA samples out was carried out by using a sense primer specific for the leader segment of the V_H gene and an antisense primer to the constant region corresponding to the tumor idiotype using conditions and primers previously described[39]. A portion (1/25th) of the first-round PCR reaction was reamplified using the same V_H leader primer and a specific CDR3 antisense primer. PCR products were analyzed by electrophoresis through 2% agarose gels containing 1 μg/ml ethidium bromide. Bands were visualized and photographed under UV light. The sensitivity of each primer set to specifically amplify tumor DNA was determined by applying the PCR technique to cDNA prepared from samples of 10^7 normal human PBMCs into which a single tumor cell was added. Amplification of cDNA from isotype and V_H gene family-matched tumor samples and normal spleen served as specificity controls. Amplification of β_2-microglobulin served as a positive control for the integrity of each cDNA preparation[39].

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